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14. ABSTRACT
Tendons are exquisite tissues that connect muscle to bone and couple muscle contraction to movement of the skeletal elements. The development of adhesions is a major complication associated with injury to muscles and tendons. Adhesions most commonly occur in intrasynovial locations and are common in Zone II flexor injuries of the hand (up to 30-40% of patients). Despite careful microscopic surgical approaches, optimal suture techniques and materials, and aggressive hand rehabilitation protocols tendon scarring/adhesions continues as an unsolved medical problem, particularly in severe in complex battlefield wounds. The scar tissues that form can severely compromise otherwise successful reconstructions of bone and other tissues. Continued outstanding progress has been made on our goal of establishing an innovative new method to prevent scar formation. This is based on the use of anti-sense oligonucleotides (ASOs) that target and inhibit the expression of TGF-beta1, Smad3, and CTGF (all members of the TGF-beta signaling pathway). Using an animal model of tendon injury and repair, biomechanical, histological, and gene expression analysis all show evidence of reduced scar formation while not impairing the strength of the tendon repair. This approach has tremendous relevance for translation to human studies to improve function in the injured soldier.

15. SUBJECT TERMS
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Introduction

Tendons are exquisite tissues that connect muscle units to bone and provide the mechanism to couple muscle contraction to movement of the skeletal elements. An important component of tendons is that they are functionally connected. Thus, scarring and formation of adhesions of a tendon in one finger impairs the normal excursion and function of the analogous tendons to the other fingers. The development of adhesions is a major complication associated with injury to muscle and tendon units. Even in simple tendon repairs without substantial soft tissue injury, adhesions are reported to be high as 30-40% and this event is so common that the development of severe scarring and adhesions could be considered a component of the normal healing process. Adhesions most commonly occur in intrasynovial locations, and are particularly common in Zone II flexor injuries of the hand. Thus, despite careful microscopic surgical approaches, optimal suture techniques and materials, and aggressive hand rehabilitation protocols tendon scarring/adhesions continues as an unsolved medical problem. The problem is worst in battlefield injuries that are characteristically complex, contaminated high-energy injuries involving both soft tissue and bone. The scarring that impairs tendon function prevents functional improvement in soldiers who have otherwise had successful reconstruction of muscle, nerve, bone, and joints. The purpose of these studies was to use an exciting new biological approach in an innovative animal model of tendon injury, repair, and scar formation to develop new approaches to prevent adhesion formation and improve the functional recovery of our wounded warriors.

Body

The Report provides an update on the research progress related to the approved tasks of the grant proposal. Outstanding progress has been made on the proposed tasked. The work was reported at the 2012 Meeting of the Orthopaedic Research Society as both an oral presentation and as a poster presentation. The work was recognized at the business meeting of the Orthopaedic Research Society since it received one of 9 awarded New Investigator Research Awards (NIRA). More than 600 abstracts were considered for this award.

Task 1 was to determine the ability of the anti-sense oligonucleotides targeting TGF-beta1 and Smad3 signaling pathways to reduced adhesions in tendon segmental grafts as measured by quantitative biomechanical testing.

Methods

Murine Tendon Injury and Repair Model: Anesthetized mice were subjected to a full-thickness laceration of the Flexor Digitorum Longus (FDL) tendon in the hindlimb and immediately repaired using 8-0 nylon sutures in a Kessler pattern. The myotendinous junction was released following procedures to avoid disruption of the repair site. The University of Rochester Institutional Animal Care and Use Committee approved the animal model. This is an established and previously published model in our laboratory.

Animal Groups and Antisense Oligonucleotide (ASO) Treatment:

Anti-sense RNA technologies: Under normal conditions cells produce small interfering (si) RNAs that inhibit protein synthesis and stimulate catabolism of target RNAs [1,2]. This general concept can be harnessed experimentally and therapeutically by the delivery of anti-sense

RNAs. Human trials are underway to regulate protein and gene expression to modify diseases such as leukemia, Duchenne Muscular Dystrophy, and inflammatory conditions [3-5].

Among the challenges with RNA therapies is the relatively unstable nature of RNAs. This field has been advanced with the development of a 2'-O-methoxyethyl chemical modification that results in resistance of the anti-sense oligonucleotides (ASOs) to nuclease degradation *in vivo* [6]. The ASOs used in the study were obtained in a material transfer agreement with Excaliard Pharmaceuticals, Inc., [7,8].

Animal Groups: C57BL/6J, 7- to 8-week-old male mice were obtained from Jackson Laboratories. After surgery, mice were randomly divided into four treatment groups: (1) Control (scrambled siRNA; 300microgram/microliter); and (2) *Smad3* ASO, (3) *Tgf-beta 1* ASO, or (4) *Ctgf* (Connective Tissue Growth Factor) ASO. Treatments were delivered by local injection using micro-syringes (Hamilton) at 3 post-operative time points (Day (D) 2, D6, and/or D12). Mice were harvested at day 14 and 21 and the limbs were prepared for biomechanical testing.

Adhesion & Biomechanics Testing:

Biomechanical testing using the Instron 8841 DynaMight™ axial servohydraulic testing system (lnstron Corporation) with measurement. Six lower hindlimbs were harvested at 14 and 21 days following repair and were rigidly fixed with a clamp and stabilizing rod from a custom adhesion testing apparatus.

FDL tendons were isolated and loaded with incremental static weights up to 19g, while digital images were taken at each increment (Figure 3A-D). The metatarsophalangeal (MTP) range of motion (ROM) was measured using ImageJ software by two blinded independent observers. Following adhesion testing, isolated FDL tendons were mounted individually onto the 8841 DynaMight axial servo-hydraulic testing system. Ultimate failure force (tensile strength)

and stiffness (slope of linear portion of load-deformation curve) were determined from force-deformation curves.

Results

We initially completed a series of control experiments to confirm the ability of the ASOs to be distributed into the tissues and to block gene expression and protein expression of the target genes. Black ink (100 microliters) (Bradley Products, Inc) was injected into the primary tendon repair site to investigate the distribution of ASOs in this model (Figure 1A). Widespread distribution of the ink was observed, showing the ability of the injected solutions containing the various ASOs to be distributed throughout the repair site. Subsequently, the various ASOs (300 µg of either Control, Smad3, TGFB1, and CTGF) were injected into repair sites at 2, 6, and 12 days after tendon repair (Figure 1B). Total RNA was extracted from four individual FDL repair tendons per treatment group and processed for real-time RT-PCR after 21 days. The gene expression of *Smad3*, *TgfB1*, and *Ctgf* was examined in the various groups (n=4). Expression was standardized with the internal beta-actin control and the relative expression of each of the individual genes is show relative to that particular control group set at 1.0. The data demonstrate that each of the ASO's significantly reduced the expression of the various target mRNAs by 40 to 60 percent. The largest inhibition occurred in *Smad3* mRNA expression upon Smad3 ASO treatment.

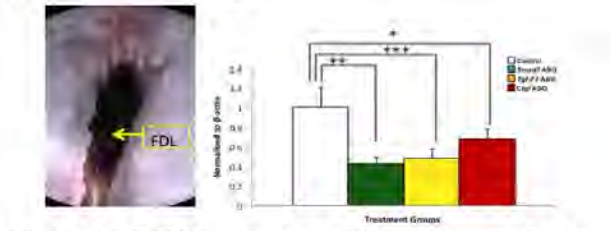

Figure 1: Defining the area of ASO exposure in the repair site and the ability of the ASOs to inhibit the expression of the targeted genes. Figure 1A: Black ink was injected into the primary repair tendon site at day 2 in 100 microliters of solution. Figure 1B: Tendon repair was performed and ASOs (control, Smad3, TGF-beta, and CTGF) were injected at days 2, 6, and 12. Total RNA was isolated from the tendons after 21 days. Statistical significance is shown by *, **, and *** which indicate significant differences of $p<0.05$, $p<0.01$, and $p<0.001$, respectively.

We examined Smad3 protein expression using immunohistochemistry. Using the same injection protocol, we performed immunohistochemistry on tissues that were harvested from

control ASO and Smad3 ASO treated mice at 7, 14, and 21 days (Figure 2). While abundant Smad3 protein expression was observed in the control ASO healing tendon tissue samples, a marked reduction in Smad3 protein was observed in the healing tendon tissue of the Smad3 ASO treated mice at day 7, 14, and 21 (Figure 2). These control experiments show that the ASOs reduce the expression of the target genes and result in reduced protein expression.

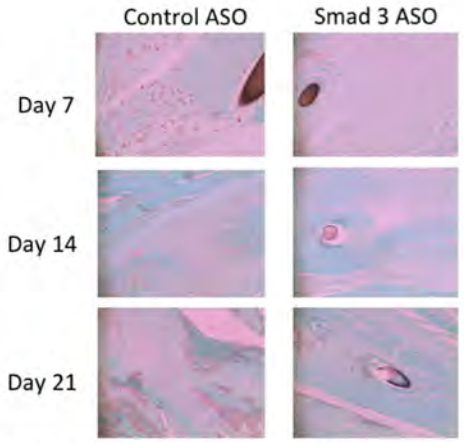

Figure 2: Smad3 Immunohistochemistry of the tendon healing area in mice with tendon repair at 7, 14, and 21 days. Either control ASO or Smad3ASO was injected into the injury and repair area a 2, 6, and 12 days after the tendon repair. Note the reduced expression of Smad3 protein in tendons that have received Smad3 ASO injection

Adhesion testing showed a significant decrease in metatarsal phalangeal (MTP) joint range of motion (ROM) in all experimental groups at D14 and D21 compared to un-operated tendon. This is consistent with the formation of scar tissue and adhesions. However, the range of motion in the MTP joints in the groups treated with the ASOs that blocked parts of the TGF-beta pathway (anti-TGF-beta1, anti-Smad 3, and anti-CTGF) was increased compared to the group treated with the control (scrambled) ASO. This indicates that the ASO treatment was able to adhesion formation following tendon injury (Figure 3).

A potential concern is that the reduction in scar formation could result in reduced stiffness and a reduction in the strength of the repair. However, no major difference in stiffness was observed between the control and experimental groups (Fig. 4A and 4B). Thus, the reduction in scar

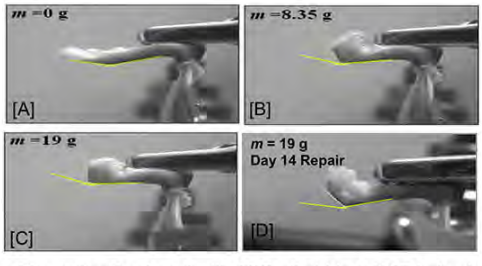

Figure 3: Treatment with ASOs that block TGF-beta signaling targets result in increased MTP joint ROM, consistent with reduced adhesions. Statistical significance is show at *p<0.05 and **p<0.001.

formation did not result in a decrease in the strength of the tendon during the repair response. We are in final preparation of these data for publication along with work presented in Task 2.

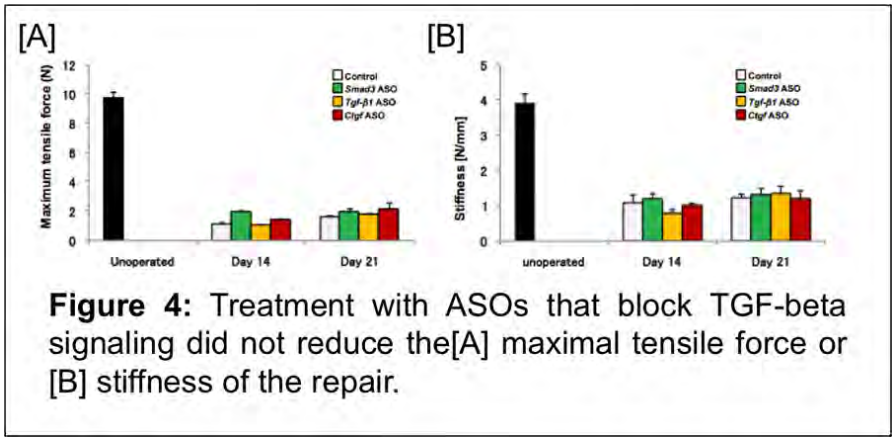

Figure 4: Treatment with ASOs that block TGF-beta signaling did not reduce the[A] maximal tensile force or [B] stiffness of the repair.

As we completed the *in vivo* experiments related to Task 1 we developed an innovative *in vitro* model of tendon scar formation that is highly complementary and which we believe has the potential to obviate the need some of the extensive and more costly *in vivo* animal experiments. In this model we have implanted tendon cells into an organ culture in a 3-dimensional collagen gel (Figure 5). In this model isolated tendon cells from mice can be expanded in primary culture and counted using a hemocytometer. The tenocytes are then pelleted, resuspended in media (MEM a supplemented with 1% FBS and 1% Pen Strep), and mixed with an isotonic, neutral collagen I solution (Advanced BioMatrix, #5005-B) at a volume ratio of 1:19 to achieve a final cell density of 70,000 cells/mL and collagen concentration of 2.3 mg/ml. The cell-seeded collagen is then cast into custom-made silicone constructs (Figure 5A) around two screws made of Polyether- etherketone (SmallParts, #B000MN4SAI), an autoclave-ready polymer, and gelled in an oven set to 37 degrees C for 1 hour. After gelation, the edges of the collagen gels were separated from the sides of the silicone construct with a sterile spatula, and 2 mL of control media was added to the media well of each construct (Figure 5A). Over time, the gels undergo contraction and this can be examined using image analysis over time (Figure 5B) or gene expression can be examined following isolation of total RNA from the gels.

To assess effect of TGF-beta, after overnight culture, control media was replaced with

fresh control media or media containing growth factor, and gel contraction and gene expression were examined. A set of gels from each treatment group (n = 3) was imaged immediately before treatment (0 hours) and again at 6, 24 and 48 hours after treatment with a digital camera (SPOT RT3). In addition, a set of gels from each treatment and time point (n = 3) was frozen at - 80℃ for RNA purification at a later time. Images of the gels is analyzed with ImageJ (available at http://rsb.info.nih.gov/ij; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD) to compute area contraction, which was determined by dividing the area of the gel at each time point by its area at 0 hours.

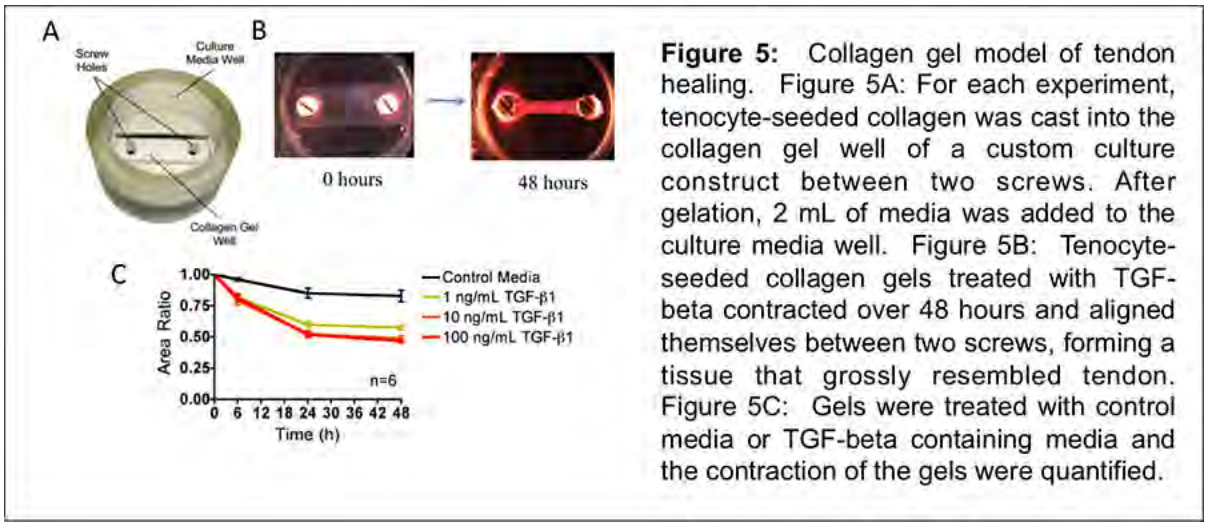

Figure 5: Collagen gel model of tendon healing. Figure 5A: For each experiment, tenocyte-seeded collagen was cast into the collagen gel well of a custom culture construct between two screws. After gelation, 2 mL of media was added to the culture media well. Figure 5B: Tenocyte-seeded collagen gels treated with TGF-beta contracted over 48 hours and aligned themselves between two screws, forming a tissue that grossly resembled tendon. Figure 5C: Gels were treated with control media or TGF-beta containing media and the contraction of the gels were quantified.

These experiments demonstrate the ability of the model to demonstrate scar formation/contraction of the gels over time. In these experiments TGF-beta causes contraction of the gels in a dose dependent manner. The experiment used duplicates for a total sample size of 6 per treatment per time point. This model provides the opportunity to assess scar formation/contraction in an in vitro collagen gel model of tendon healing. The findings are consistent with TGF-beta increasing scar formation. Additional data with the collagen gel that is presented with Task 2 shows that TGF-beta increases the expression of collagens in healing model. The findings complement our animal experiments in which blocking TGF-beta reduces scar formation and adhesions. The collagen gel model will enable us to further explore the

relative effectiveness of the various TGF-beta signals and targets as inhibitors of scar formation through administration of ASOs.

Task 2 was to demonstrate the ability of anti-sense oligonucleotides targeting TGF-beta, Smad3, and CTGF to modulate the expression of collagens and MMPs involved in the anabolic and catabolic response to tendon injury and result in reduced scar formation as measured by histology, *in situ* hybridization, gene expression, Western blot, and zymography.

Methods

Murine Tendon Injury and Repair Model, animal groups, and ASOs: See description in Task 1.

Histology: Specimens were harvested at several time points after surgery and ASO treatment (Day 3, 7, 10, 14, or 21) and paraffin embedded.

Quantitative Real-time RT-PCR: Four FDL tendons were harvested per group at five time points (D3, D7, D10, D14, and D21). Total RNA was extracted from each using the RNAeasy Lipid tissue kit (Invitrogen). Single-stranded cDNA was made using a reverse transcription kit (BioRad). Using murine specific primers for target genes (*Smad3, Tgfb1, Ctgf),* as well as markers for scar formation *(Col1a, Col3a)* and tendon formation (*Scleraxis* (*Scx*)*, Tenomodulin* (*Tnmd*)). mRNA expression was measured and standardized to *ß-actin*. Data were normalized to un-operated wild-type tendons, which served as a pre-operative time point (D0).

Results

Histology: has clearly revealed decreased fibrous tissues surrounding the repair site in all ASO treatment groups when compared with control group at D7, D14, and D21 (Fig. 6). These results are consistent with reduced scar formation and adhesion in the tendon repairs treated with ASOs that target TGF-beta1, Smad 3, and CTGF.

Gene expression: studies were performed on the tissues between lacerated tendons. The gene expression studies are consistent with a healing process that favors the regeneration of actual tendon tissue as opposed to the formation of scar. *Scleraxis* (*Scx*), which expressed

specifically in tendon during development and repair, is upregulated in the tendon repair cells in which there is suppression of the TGF-beta signaling pathway genes through ASO treatment (Figure 7).

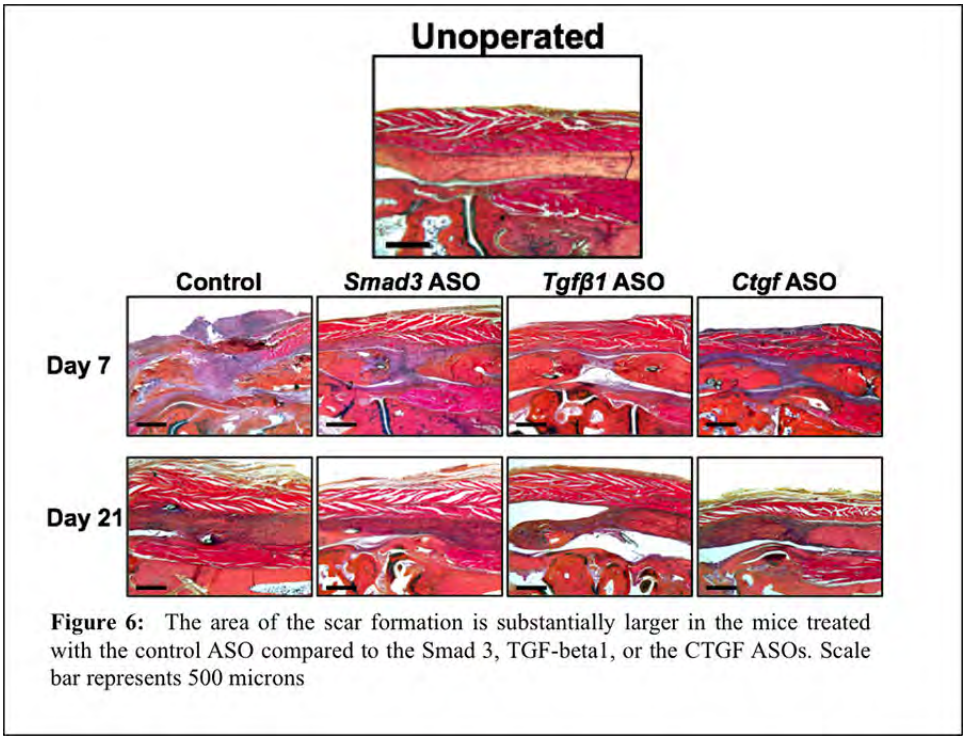

Figure 6: The area of the scar formation is substantially larger in the mice treated with the control ASO compared to the Smad 3, TGF-beta1, or the CTGF ASOs. Scale bar represents 500 microns

Injured tissues make scar forming granulation tissue that is characterized by high levels of type III collagen (*Col3*), which results in a less organized tissue structure. More mature tissues, including tendon, are composed of type I collagen (*Col1*). Consistent with reduced adhesions upon ASO treatment (see Task 1), scar tissue, and the formation of a more mature tissue, the ASO treated repairs that target TGF-beta signaling pathways have reduced expression of *Col1* and increased expression of *Col3* (Figure 8). In particular Realtime RT-PCR at day 10 showed a significant decline in *Col3* expression, which is typically related with the development of scarring after tendon injury, in all

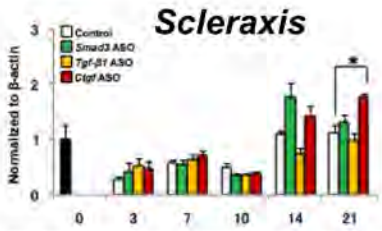

Figure 7: The expression of *Scleraxis* is increased in the tendons repairs in mice that are treated with ASOs that target Smad 3, TGF-beta1, and CTGF.

ASO treatment groups. At day 21 there is a marked increase in the expression of *Col1* expression, which is consistent with mature tendon formation.

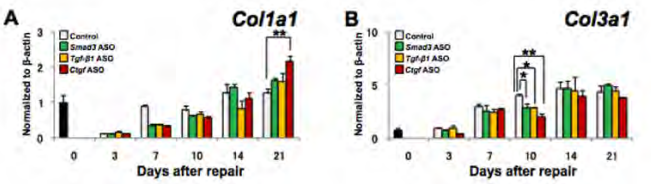

Figure 8: Gene expression of type I collagen (A) and type III collagen (B) in tendon repair. Treatment with ASOs targeting Smad 3, TGF-beta1, and CTGF result in reduced type III collagen expression and increased type I collagen expression, consistent with regeneration of more mature tendon tissue in the treated animals.

We also examined the expression of matrix metalloproteinases (MMPs). MMPs have a major role in the remodeling of tissues, particularly tissues undergoing repair and are necessary for the formation of a highly organized tissue. *Mmp9* has previously been shown by published work in our laboratory to be important in tendon repair. The pattern of *Mmp9* expression is altered in the ASO treated tissues. Compared to control (scrambled) ASO, the ASOs that target TGF-beta1, Smad 3, and CTGF tend to reduce *Mmp9* expression early (day 7 and 10; parallel to the reduced expression of *Col3*) and have increased expression later (day 21; during the period of increased expression of type I collagen) (Figure 9). The effect is greatest with TGF-beta ASO treatment.

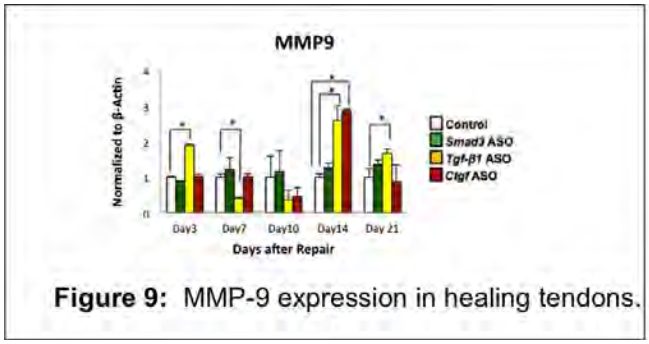

Figure 9: MMP-9 expression in healing tendons.

The collagen gel model of tendon healing was examined for gene expression (See Figure 5). Mouse tendon cells were cultured in collagen gels and treated with either control media or media containing various concentrations of TGF-beta. In contrast to the early in vivo reduction in the expression of the *Col1* and *Col3* genes in healing tendon with TGF-beta inhibition, the treatment of the collagen gels with TGF-beta resulted in increased expression of *fibronectin, Col1,* and *Col 3*. This is consistent with the role of TGF-beta in the increase in scar formation.

Key Research Accomplishments (in murine model of tendon healing):

1. Demonstrating the ability of ASO s to be delivered to healing tendon tissue that target the expression of specific genes;
2. Demonstrating the ability of ASOs to reduce gene expression and protein expression of target genes in healing tendon tissue.
3. Demonstrating the ability of ASO's directed against TGF-beta1, Smad 3, and CTGF (all of which are components of the TGF-beta signaling pathway) to reduce adhesions during tendon healing as measured by biomechanical testing;
4. Establishing that the reduction of adhesions through treatment of healing tendons with ASOs does not result in reduced tendon strength or stiffness.
5. Demonstrating the ability of ASOs directed against TGF-beta1, Smad 3, and CTGF to reduce the formation of scar tissues as determined by histologic analysis;
6. Demonstrating the ability of the ASOs targeting TGF-beta1, Smad 3, and CTGF to stimulate increased expression of tendon specific cell markers in healing tendon tissues.
7. Demonstrating the ability of the ASOs targeting TGF-beta1, Smad 3, and CTGF to alter the pattern of collagen expression (reduced type III collagen and increased type I collagen) consistent with the formation of a more mature/organized healing tissue.
8. Demonstrating the ability of ASOs targeting TGF-beta to modulate the expression of MMPs in the healing tendon tissue.
9. Demonstrating the ability of an innovative collagen gel model of tendon healing to undergo contraction.
10. Demonstrating the ability to measure gene expression in the collagen gel model of tendon healing.
11. Demonstrating the ability of TGF-beta to increase collagen expression and induce contraction/scarring in the collagen gel model of tendon healing.

Reportable Outcomes

1. Orthopaedic Research Society Abstract, Presentation, and Award: An abstract on this work was submitted to the Orthopaedic Research Society and accepted for the 2012 meeting that was held in San Francisco, CA in February of 2012. Prior to the meeting, the abstract was selected from more than 600 abstracts as among 35 finalists for a New Investigator Research Award. At the meeting, based on an oral presentation and a poster presentation, the work was awarded one of the nine New Investigator Research Awards presented by the Orthopaedic Research Society. This manuscript is being prepared for submission.

   Kondabolu SK, Yukata K, Shi S, Jonason J, Li TF, Awad HA, O'Keefe RJ. "Development of siRNA Technology to Prevent Scarring and Adhesion Formation after Flexor Tendon Repair." Transactions of the 2012 Orthopaedic Research Society Annual Meeting, p.73. ORS 2012 Feb.

2. National Institute of Health (NIH) CTSI K22 Grant Award: Sirish Kondabolu, a medical student at the University of Rochester took a year out of his medical school studies in 2010-2011 to work on this project. Mr. Kondabolu applied for and received a K22 Award from the University of Rochester NIH funded CTSI research program to support student education and training through work on this project. Mr. Kondabolu has had the opportunity to present this work at CTSI research seminars and meetings. Mr. Kondabolu was awarded the ORS NIRA award for this work.

3. Dr. O'Keefe was the keynote speaker at the Orthopaedic Research and Education Foundation Resident Research Conference for the Southwestern United States that was

jointly sponsored by the San Diego Naval Medical Center and the University of California San Diego Orthopaedic Programs (May 24 and 25, 2011). The presentation focused on this work and the importance of basic and translational research approaches for the improvement of care for out military personnel.

Conclusions

The development of adhesions is a major complication associated with tendon repair with an occurrence as high as 30-40%. Inflammation associated with injury is a strong stimulus for scarring. The scarring and formation of adhesions of a single tendon one finger impairs the normal excursion and function of the analogous tendons to the other fingers. The formation of scar tissue compromises the outcomes of successful regeneration of bone soft tissues, nerve, and other structures.

The present studies provides innovative new materials and approaches that reduce inflammation and scarring, optimize the tendon healing environment, enhance mobilization, and reduce surgical complications and morbidity. The innovation involves the use of anti-sense gene approaches and technologies to target specific proteins and signaling molecules to reduce their expression. In particular the approach targeted the TGF-beta signaling pathway.

TGF-β signaling has been shown to be a key regulator of scarring and fibrosis in numerous tissues and disease processes, including pulmonary fibrosis, nephrosclerosis, hypertrophic skin scarring, liver fibrosis, and other diseases [7-11]. TGF-β is implicated in the scar formation that occurs in the setting of injury, including the scar formation that occurs following myocardial infarction [12]. TGF-β has been shown to be induced early in the process of healing in several tendon injury and repair models, including rabbit, horse, canine, and chicken flexor tendon injury and repair models [13,14 15 16,17]. TGF-β enhances scarring through numerous mechanisms, including stimulation of fibroblast proliferation and migration, collagen and fibronectin synthesis, and altered tissue remodeling through regulation of MMPs [18,19].

The present work has provided and important proof of principle using a relevant animal model. The key research accomplishments are listed in the appropriate section (see Key Research Accomplishments section). However, in aggregate the data, through experimental methods that assess a variety of aspects of tendon healing show that the Anti-sense

Oligonucleotide approach to gene inhibition is effective in reducing scar formation and improving functional of the healing tendon. Furthermore, the approach did not result in decreased repair strength.

We have provided extensive evidence that flexor tendon injuries can be enhanced through simple treatment with ASOs. This approach has high clinical translantion and could have a major impact on the care of the wounded warrior and the civilian population.

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Appendices and Supporting Data: All relevant data appears in the body of the report.